THE STRUCTURE OF TOBACCO MOSAIC VIRUS AND ITS COMPONENTS: ULTRAVIOLET OPTICAL ROTATORY DISPERSION

NORMAN S. SIMMONS *and* ELKAN R. BLOUT

From The Laboratory of the Children's Cancer Research Foundation and Harvard Medical School, Boston, and The Department of Biophysics and Nuclear Medicine, Laboratory of Nuclear Medicine and Radiation Biology, The University of California, Los Angeles School of Medicine, Los Angeles. Dr. Blout's alternate address is Research Division, Polaroid Corporation, Cambridge, Massachusetts

ABSTRACT An investigation has been made of the optical rotatory dispersion in the region 226 to 366 mμ of tobacco mosaic virus (TMV), the protein subunits isolated therefrom, the rods synthesized from the protein subunits, and the ribonucleic acid (RNA) isolated from TMV. Both TMV and the protein rods show anomalous rotatory dispersion. The RNA shows a Cotton effect with an inflection point around 260 mμ, which is shifted to 272 mμ in concentrated urea solution. A suggested interpretation of the RNA rotatory dispersion is given. The rotatory dispersion of the protein subunits shows an incipient Cotton effect with an inflection point around 293 mμ and the beginnings of a large negative Cotton effect with a trough at 232 mμ. The dispersion data from the protein subunits can be interpreted to indicate that they contain between 25 and 35 per cent α-helix. On the basis of recent sequence investigations and the relationship between amino acid composition and polypeptide structure, the helical portion of the protein subunits can be located in the central section of the protein chain.

INTRODUCTION

As a result of extensive x-ray diffraction, chemical, and physical-chemical studies, tobacco mosaic virus (TMV) has been shown to be a hollow cylinder of helically arranged asymmetric protein subunits containing within them a single ribonucleic acid (RNA) chain which winds between turns of the protein helix. A complete review of the known structural parameters of TMV has recently been prepared by Klug and Caspar (1).

This work was supported in part by United States Public Health Service Grant A2558 and by Contract No. AT(04-1)-GEN-12, between the Atomic Energy Commission and the University of California.

Received for publication, August 15, 1960.

The protein subunits may be isolated free of RNA (2) and reversibly aggregated into hollow, cylindrical rods as a function of temperature, pH, and ionic strength (3). X-ray diffraction studies indicate that these RNA-free protein rods have an internal structure almost indistinguishable from that of the protein shell of the original virus (4).

X-ray diffraction and birefringence studies have indicated the RNA chain to be coiled between turns of the helical protein shell and the planes of the purine and pyrimidine bases aligned approximately parallel to the fiber axis of the virus (5). Since the pitch of the virus helix is 23 A, there is no possibility of hydrogen bonding between bases on successive turns of the helix and the structure of the RNA in the TMV will collapse when the protein is removed. Thus any secondary structure of isolated RNA must be due to base interactions that do not exist in the RNA contained in the virus. The fact that there is secondary structure in isolated TMV-RNA is indicated by ultraviolet absorption spectra and visible optical rotation measurements (6, 7).

The amino acid composition of the protein subunit has been known for some time and the sequence of these amino acids has been recently determined (8), but there is little information available as to the structure or conformation of the protein chain itself (9, 10). The most direct evidence on the structure of the protein subunit is that obtained from studies of the infrared dichroism of oriented TMV. These studies (9, 10) indicate that the protein subunits contain α-helices oriented radially to the long axis of the tobacco mosaic virus.

Since it is now possible to characterize certain asymmetric macromolecular structures by optical rotatory dispersion we have determined the dispersion characteristics of the whole virus, the RNA-free repolymerized protein rods, and the individual protein subunits as well as the isolated RNA. This paper presents the data which show (*a*) that the "native" TMV-protein subunits have in themselves a characteristic optical rotatory dispersion indicating a considerable helix content; (*b*) that this characteristic dispersion is altered markedly by polymerization of the subunits into protein rods—a "superhelix"; (*c*) that the isolated RNA shows rotatory dispersion with a Cotton effect having an inflection point at about 260 mμ, the significance of which will be discussed below.

EXPERIMENTAL

Materials

Tobacco Mosaic Virus (TMV). Leaves of *Nicotiana tabacum* var. *Turkish Samsun,* infected with the U_1 strain (11), were harvested and frozen. The partially thawed leaves were homogenized with distilled water and deionized with a mixture of weak base resin (amberlite CG-45) and weak acid resin (amberlite CG-50). The pulp and resin were removed from the extract by centrifugation and the supernate made 0.01 M with versene at pH 7.3. The virus was isolated by differential centrifugation (12) from 0.01 M

versene three times. The clear colorless virus pellet was finally taken up in water to a concentration of 2 per cent and dialyzed to remove residual salt.

TMV-PROTEIN SUBUNITS. The protein subunits of TMV were isolated with minor improvisations according to the method of Fraenkel-Conrat (2) using cold 66 per cent acetic acid. Ultracentrifugal analysis showed the protein to have a sedimentation constant of 4 and to be essentially homogeneous.

FORMATION OF PROTEIN RODS. The protein rods were formed from the protein subunits of TMV by the addition of NaH_2PO_4 to a concentration of 0.01 M. On ultracentrifugal analysis these rods showed a hypersharp peak with a sedimentation constant of about 200, remarkably similar to the original whole virus. Also, x-ray diffraction showed these synthesized rods to be essentially similar in internal structure to the whole tobacco mosaic virus with the exception, of course, of the absence of the RNA (13).

PREPARATION OF THE TMV-RIBOSE NUCLEIC ACID (TMV-RNA). The RNA was isolated from the virus after the method of Cohen and Stanley (14). 5 ml of 0.10 per cent TMV in 0.1M phosphate buffer, pH 7.2, was rapidly pipetted into a 13 x 150 mm test tube (containing a quarter-inch teflon-covered magnetic stirring bar) immersed in a water bath maintained at 80° over a magnetic stirrer hot plate. The solution became turbid with coagulated denatured protein in about 45 seconds. After a total of 60 seconds the tube was removed from the bath and immediately plunged directly into an ice bath similarly mounted on another magnetic stirrer. Coagulated protein was removed by centrifugation at 7,000 R.P.M. in a Servall at 4°. The water-clear supernate showed a typical ultraviolet RNA spectrum with a maximum at 259 mμ and a minimum at 230 mμ, indicating almost complete absence of contaminating protein. The yield was almost invariably 100 per cent of the RNA contained in the virus.

DENATURED PROTEIN SUBUNITS AND RNA. Aliquots of the protein subunits and RNA were made 8 M with recrystallized urea and held at 60° for 1 hour and then cooled to room temperature. All further dilutions for rotation measurements were made with 8 M urea. The 8 M urea solutions themselves showed no rotation.

Method

Optical rotations were measured with a Rudolph photoelectric polarimeter, model 200 using a General Electric AH-6 water-cooled high pressure mercury arc as the light source. The lamps were selected for maximum output below 240 mμ. A Beckman DU monochromator was used to obtain essentially monochromatic radiation. Since the measurements were made in the region from 366 mμ down into the far ultraviolet the quartz lens that focuses the lamp into the monochromator was adjusted so as to achieve maximum light intensity at 297 mμ. All solutions were measured in fused quartz cells of 1 cm path length selected to have minimum birefringence. All measurements were made at a symmetrical angle of 5°. Because of the high absorbance of these solutions in this wavelength region it was necessary to make careful adjustments of solute concentrations to get maximum rotation readings consistent with their absorbance characteristics. This meant taking a set of measurements for a given solute concentration over a small spectral increment.

The data are expressed in terms of specific rotation which is defined as $[\alpha] = 100\alpha/1 \cdot c$, where α = the observed rotation in degrees, and c = the concentration of the solute in grams per 100 ml solution and 1 = cell path (in decimeters). Because of the high absorbance and the necessity for working with dilute solutions, the measured rotations were very small and frequently below 0.1°. We estimate that our maximum uncertainty

for a reading at a particular wavelength in these regions is ±0.01° with an internal constancy of ±0.005°.

RESULTS AND DISCUSSION

TMV-PROTEIN SUBUNITS. The rotatory dispersion data of the protein subunits over the wavelength range 226 to 366 mμ are shown in Fig. 1. It is apparent that this protein shows anomalous rotatory dispersion and therefore the data do not fit

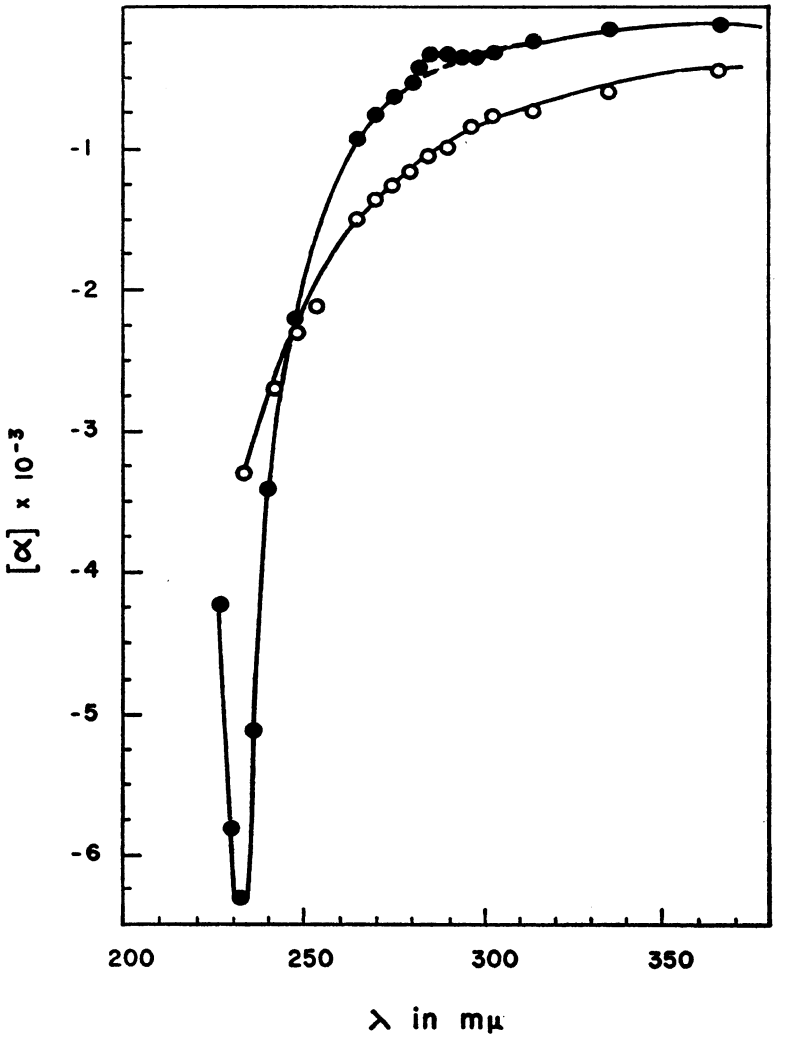

FIGURE 1 ●-●-●-●-, the rotatory dispersion of protein subunits from tobacco mosaic virus in distilled water, o-o-o-o-, the denatured form of the protein subunits of tobacco mosaic virus in 8 M urea solution.

a simple Drude equation. Over the wavelength region 365 to 600 mμ the rotation data do fit the two term relation suggested by Moffitt (15) and from this relation the coefficient of the second term b_0 has been calculated to be equal to −160. Assuming that fully α-helical polypeptides and fibrous proteins have b_0 values about 600 (16) then, on this basis, it appears that the protein subunits of TMV contain about 25 per cent α-helix.

Another indication that the protein subunits contain a helix is the fact that the beginning of an ultraviolet Cotton effect is observed with a trough at 233 mμ. This Cotton effect has recently been found to be characteristic of the α-helical form of certain synthetic polypeptides and fibrous proteins (17). When the protein subunits are denatured this ultraviolet Cotton effect is lost (Fig. 1). Furthermore it should be noted that the amino acid sequence analysis (8) indicates that both terminal positions of the protein chain contain large sequences of non-helix-forming amino acid residues (18). Thus it is clear that the helical part of the protein chain resides in the central portion.

One additional feature of the rotatory data of the native protein subunits is the presence of a small inflection point—perhaps an incipient Cotton effect—around 293 mμ. This is the first such effect observed for a native protein but detailed investigation of it will require improved instrumentation. The region around 280 to 290 mμ is where such a Cotton effect would be observed if oriented aromatic amino acids exist in the TMV-protein subunits. It is possible that this effect can be assigned to oriented tryptophan or phenylalanine residues since oriented tyrosyl groups show a Cotton effect at shorter wavelengths (19).

PROTEIN RODS. When the protein subunits of TMV are polymerized to form helical protein rods, the optical rotation changes to more positive values and there is essentially no rotation above 270 mμ (Fig. 2). This change in rotatory dispersion, a consequence of the formation of the helical rods, cannot be precisely interpreted at this time.

TOBACCO MOSAIC VIRUS (TMV). The rotatory dispersion of native TMV over the wavelength range 230 to 366 mμ is shown in Fig. 2. The data indicate that the dispersion is anomalous (in that the rotation changes from positive to negative). The reason for this anomalous dispersion of course lies in the asymmetry of the virus structure. Since the rotatory dispersion of TMV is quite different from that of the TMV-protein rods, the assumption is that this difference is due to the RNA moiety.

It is noted, however, that the TMV itself shows strongly positive rotation above 275 mμ with a maximum at approximately 285 mμ. This strong positive contribution must be assigned to the presence of the RNA helix in the intact virus. Detailed examination of the dispersion curves of the TMV and the TMV-protein rods from 270 to 236 mμ may, therefore, similarly be interpreted as being due to the presence of the RNA in the intact TMV. If a mathematical subtraction of the TMV-protein

rod dispersion data from those of the intact TMV is performed, a calculated dispersion curve attributable to the RNA, shown in the dashed line in Figs. 2 and 3, is obtained.

ISOLATED TMV-RNA. In Fig. 3 the rotatory dispersion of TMV-RNA can be seen to be anomalous and in fact shows a Cotton effect at 260 mμ, the region of absorption of the component purine and pyrimidine bases.

The presence of a Cotton effect with its inflection point at the absorption maximum of the component bases indicates that these bases are involved in an asymmetric conformation. To ascertain whether the magnitude and position of the Cotton effect reflect interaction between oriented H-bonded purines and pyrimidines, or are due only to the asymmetric environment around the optically active ribose moiety, we have attempted to disrupt any such bonding by treatment with 8 M urea. The data are shown in Fig. 3 where it can indeed be seen that there is a marked change in the dispersion curve obtained in urea solution as compared with same

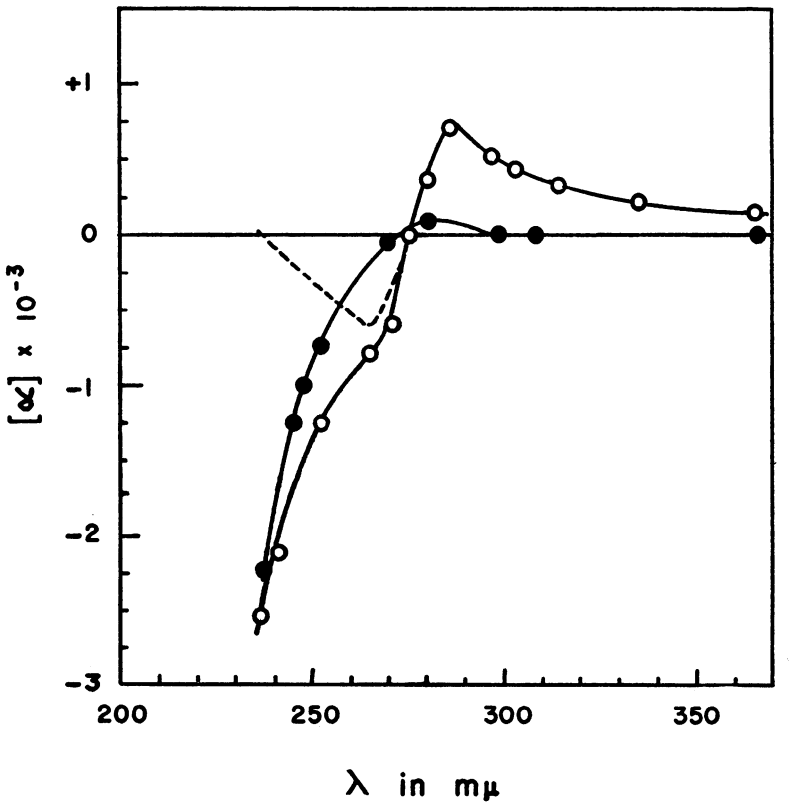

FIGURE 2 o-o-o-o-, the rotatory dispersion of tobacco mosaic virus in distilled water. ●-●-●-●-, the rotatory dispersion of the protein rods synthesized from the tobacco mosaic virus-protein subunits in 0.01 M NaH_2PO_4. - - - -, the calculated curve obtained by subtracting the rotatory dispersion of the protein rods from that of the tobacco mosaic virus.

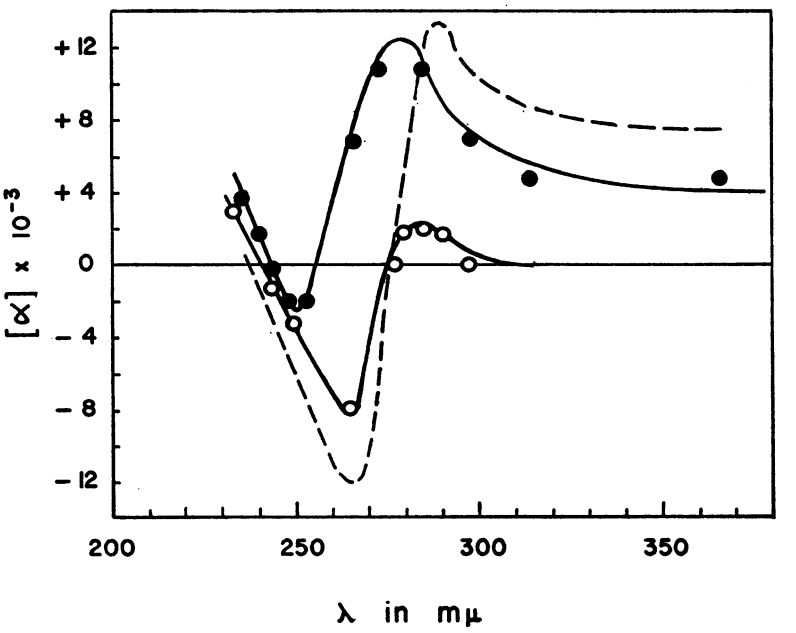

FIGURE 3 •-•-•-•-, the rotatory dispersion of ribonucleic acid in phosphate buffer isolated from TMV as described in the experimental section. o-o-o-o-, the rotatory dispersion of ribonucleic acid from tobacco mosaic virus in 8 M urea. - - - -, the calculated curve (multiplied by 20 because the RNA content of TMV $\simeq$ 5 per cent) obtained by subtracting the rotatory dispersion of the protein rods from that of the tobacco mosaic virus.

material in water. The curve has been shifted toward longer wavelengths with a concomitant diminution of the positive rotatory portion. Exciton theory (15, 20) indicates that a shift toward longer wavelengths results from the disruption of (intramolecular hydrogen bonding between) card-stacked chromophores whereas a shift towards shorter wavelengths results from disruptions of end-to-end-bonded chromophores. We thus conclude that there may be some planar (card-stacked) base-to-base interaction in isolated RNA in water solution.

It is important to note that the "calculated" RNA rotatory dispersion curve shows approximately the same inflection point (270 to 275 mμ) as the urea-treated RNA. Thus the rotatory dispersion data on RNA can be interpreted as indicating the lack of planar base-base bonding of the RNA in the native TMV in agreement with the conclusions from x-ray studies, and the presence of base-base interaction in isolated RNA in water solution. The magnitude of the "calculated" RNA dispersion curve suggests that the bases are indeed rigidly oriented in TMV as suggested by the strong positive birefringence. Taken as a whole the physical-chemical evidence indicates that in TMV the RNA exists in a rigid structure which might involve bonding of the base to sites on the protein shell or through the medium of bound water.

CONCLUSIONS

The most important conclusion from the optical rotatory dispersion data is that the helix content of the protein subunits can be estimated as being between 25 and 35 per cent. This estimate of helix content is based on both visible and ultraviolet optical rotatory dispersions. Although the rotatory dispersion changes markedly when the protein subunits are polymerized into protein rods, no interpretation of these data can be made at this time. However, from the optical rotatory dispersion data of the intact virus and those of the protein rods, a "calculated" rotatory dispersion curve of the RNA in the virus has been obtained. This rotatory dispersion curve is different from that obtained with the isolated RNA, both of which show Cotton effects in the ultraviolet region. From the fact that the inflection point of the rotatory dispersion curve of the isolated RNA in concentrated urea solution approximates that of the "calculated" dispersion curve, it is concluded that there is no interaction between bases of the RNA in the native TMV.

We are pleased to acknowledge several interesting discussions with Drs. D. L. D. Caspar and Carolyn Cohen.

REFERENCES

1. KLUG, A., and CASPAR, D. L. D., *Advances in Virus Research*, **8**, in press.
2. FRAENKEL-CONRAT, H., *Virology*, 1957, **4**, 1.
3. SCHRAMM, G., and ZILLIG, W., *Z. Naturforsch.*, 1955, **10 b**, 493.
4. FRANKLIN, R. E., *Biochim. et Biophysica Acta*, 1955, **18**, 313; *Nature*, 1956, **177**, 929.
5. FRANKLIN, R. E., CASPAR, D. L. D., and KLUG, A., *in* Plant Pathology: Problems and Progress, 1908-1958, (C. S. Holton, editor), Madison, University of Wisconsin Press, 1959, 447.
6. GIERER, A., *Nature*, 1957, **179**, 1297; *Z. Naturforsch.*, 1958, **13 G**, 477.
7. BOEDTKER, H., *Biochim. et Biophysica Acta*, 1959, **32**, 519.
8. ANDERER, F. A., UHLIG, H., WEBER, E., and SCHRAMM, G., *Nature*, 1960, **186**, 922.
9. FRASER, R. D. B., *Nature*, 1952, **170**, 491.
10. BEERS, M., *Biochim. et Biophysica Acta*, 1958, **29**, 423.
11. Kindly supplied by Professor S. G. Wildman, Department of Plant Pathology, University of California, Los Angeles.
12. SIEGEL, A., and WILDMAN, S. G., *Phytopathology*, 1954, **44**, 277.
13. HOLMES, K. G., and CASPAR, D. L. D., personal communication.
14. COHEN, S., and STANLEY, W. M., *J. Biol. Chem.*, 1942, **144**, 589.
15. (*a*) MOFFITT, W., *J. Chem. Physics*, 1956, **25**, 467. (*b*) MOFFITT, W., *Proc. Nat. Acad. Sc.*, 1956, **42**, 736. (*c*) MOFFITT, W., FITTS, D. D., and KIRKWOOD, J. C., *Proc. Nat. Acad. Sc.*, 1957, **43**, 723.
16. For original references see Blout, E. R., Polypeptides and proteins, *in* Optical Rotatory Dispersion, (C. Djerassi, editor), New York, McGraw-Hill Book Company, 1960, 238.
17. SIMMONS, N. S., COHEN, C., SZENT-GYORGYI, A. G., WETLAUFER, D. B., and BLOUT, E. R., data to be published.
18. BLOUT, E. R., DE LOZÉ, C., BLOOM, S. M., and FASMAN, G. D., *J. Am. Chem. Soc.*, 1960, **82**, 3787.
19. FASMAN, G. D., and BLOUT, E. R., data to be published.
20. MCRAE, E. G., and KASHA, M., *J. Chem. Physics*, 1958, **28**, 721.